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NUCLEOTIDE VACCINE COMPOSITION

TECHNICAL FIELD

The present invention generally refers to vaccine compositions and in particular to a nucleotide vaccine composition and use thereof.

BACKGROUND OF THE INVENTION

Ever since Edward Jenner's discovery of using cowpox viruses for the prevention of the deadly smallpox in the late 18th century, vaccination has been widely employed to prevent sever infectious diseases, including smallpox, polio, tetanus and measles, in several countries around the world.

Traditionally, a vaccine is produced by inactivating or attenuating the pathogens. In this process, the pathogenic microorganism hopefully becomes harmless as a biological agent but still remains its immunogenicity. Injection of such inactivated or attenuated microorganism into a host will elicit an immune response that is capable of preventing subsequent infections with a vital, i.e. non-attenuated, pathogenic microorganism.

A major concern with these traditional vaccines is whether all of the injected microorganisms actually are inactivated. If not correctly attenuated the microorganism might start multiplying, leading to serious consequences for the host receiving the vaccine. In addition, the immunity achieved based on vaccines with inactivated microorganism is often incomplete, short lived and requires multiple vaccinations, which are far from optimal. Furthermore, the attenuation or inactivation process might alter the antigens of the microorganism, leading to redundancy of the immunogenicity of the antigens.

An alternative vaccination method based on nucleotide sequences, including DNA or RNA sequences, has been developed during the last decade. In this

vaccination strategy only those components that are relevant as immunological material are used and not the complete pathogenic (attenuated) microorganism. In the host, the nucleotide sequences are expressed resulting in antigenic protein or peptide fragment production that will elicit the host's immune response.

The DNA-based vaccination approach to elicit antigen-specific immune responses has been rapidly developed since the early 1990s. The first demonstration of the protective efficacy of a DNA vaccine showed that DNA immunization with an influenza nucleoprotein resulted in the generation of nucleoprotein-specific cellular immune responses and protection from a subsequent challenge with heterologous influenza strains [1].

DNA immunization has been studied in animal models against various infectious pathogens and malignancies [2, 3]. Immune responses to infectious antigens encoded by and delivered as plasmid DNAs have been raised in a variety of species including chickens, mice, ferrets, cattle and nonhuman primates. These infectious antigens are influenza virus hemagglutinin, matrix protein and nucleoprotein; human immunodeficiency virus 1 (HIV-1) gp120 and gp160; bovine herpes virus gIV; rabies virus surface glycoprotein; hepatitis B virus (HBV) surface antigen; Hepatitis C virus core antigen, malaria circumsporozoite protein; *Mycobacterium tuberculosis* heat shock protein 65 (Hsp 65) and antigen 85; and *Mycoplasma pulmonis* A7-1 and A8-1 antigen [4].

DNA vaccination has been shown to suppress autoimmune diseases and to inhibit allergic responses [5-7]. Recent studies of DNA vaccines demonstrated the generation of a cellular immune response against malaria infection and HIV peptides in humans revealed the utility of DNA immunization for clinical applications [8, 9]

The primary conventional treatments for malignant tumors include surgery, chemotherapy, bone marrow transplantation and radiation. Although much

progress has been made, these approaches have sometimes only a palliative effect. Alternative therapies to prevent or to treat malignant tumors are needed.

The identification of novel tumor antigen genes has promoted the expansion of DNA vaccination strategies. Direct inoculation of plasmid DNA encoding tumor-associated antigens has revealed the potential of DNA immunization for cancer therapy. The prophylactic DNA vaccination may provide one opportunity for the prevention of tumor development in individuals with genetic predisposition.

One proposed DNA vaccination strategy is the direct inoculation of plasmid DNA encoding tumor-associated antigens. Typically, plasmid DNA vaccine has two major units: (1) a plasmid backbone that delivers adjuvant and mitogenic activities via immunostimulatory sequence and (2) a transcriptional unit comprising a promoter, antigen nucleotide sequence and poly-adenylation addition sequence, which together direct protein synthesis.

Although the DNA immunization approaches discussed above have been shown to be useful in animal models, the therapeutic benefit in humans is, however, far from optimal. Improvement of vaccine efficiency has therefore become a critical goal in the development of DNA vaccinations.

Zöller M, et al., has compared different vaccination methods in a murine renal cell carcinoma model. They have shown that boosting with β-galactosidase loaded dendritic cells (DCs) in tumor-bearing mice after the oral vaccination with *Salmonella typhimurium* transformed with a plasmid DNA containing Lac Z gene results in approximately 50 % tumor free mice [10].

Kontani K, et al., has shown that injection of plasmid DNA encoding MUC1 antigen and *in vitro* cytokine activated DCs resulted in suppressing tumor growth and somewhat prolonged survival in EL4-muc tumor-bearing mice.

However the authors failed to demonstrate a therapeutic efficacy in this study since only approximately 20 % mice became tumor free after the treatment [11].

Although the above-identified vaccination protocols improve the efficiency of DNA vaccines somewhat, they are still inefficient as therapy for treatment and prevention of cancer.

SUMMERY OF THE INVENTION

It is a general object of the present invention to provide a novel nucleotide vaccine composition.

It is another object of the invention to provide a vaccine composition comprising an antigen encoding nucleotide sequence and modified antigen presenting cells.

Yet another object of the invention is to provide a vaccine composition with potent therapeutic efficacy.

A further object of the invention is to provide a vaccine composition usable for preventing and/or treating different diseases and disorders.

It is a particular object of the invention to provide a vaccine composition usable for preventing and/or treating cancer and infectious diseases.

These and other objects are met by the invention as defined by the accompanying patent claims.

Briefly, the present invention involves a novel vaccine composition comprising a nucleotide sequence encoding an antigenic molecule and gene-modified antigen presenting cells (APCs), preferably provided as an intermixture of the nucleotide sequence and the gene-modified APCs.

The antigen encoding nucleotide sequence could be a naked DNA or RNA sequence that, when adminsitered to a subject, is taken up by the subject's cells. Once taken up, the nucleotide sequence is transcribed and translated (DNA) or translated (RNA) resulting in production of the desired antigen.

However, the antigen encoding nucleotide sequence is, in an embodiment of the invention, preferably included in a vector, where the nucleotide sequence is provided under transcriptional control of a promoter. The vector of the invention is preferably a plasmid DNA vector encoding the antigen. The vector may preferably also comprise other nucleotide sequences, effecting the immune response of a subject, preferably mammalian subject and more preferably human subject, receiving the vaccine composition. Such an immune response effecting sequence could be a gene sequence coding for xenogenic molecules (proteins/peptides) participating in enhancing or suppressing the immune response of the subject.

The APCs of the vaccine composition of the present invention are cells adapted for presenting antigens to other cells, especially to CD4+ and CD8+ T cells, of the immune system. Thus, the APCs are preferably rich in surface proteins, e.g. major histocompatibility complex (MHC) class I and II, used for presenting antigens and activating T cells. Examples of preferred APCs according to the invention are dendritic cells (DCs), macrophages, monocytes and B cells.

Furthermore, the APCs are genetically modified, or otherwise modified, for expressing immune-response effecting molecules. Such molecules could enhance the immune response of a subject by increasing antigen presentation, activating the APCs and/or enhancing the immune function of APCs. Alternatively, especially for autoimmune diseases, the molecule could be suppressing the immune response of the subject. In either case, genetic material is preferably introduced into the APCs for transiently expression from an episomal location or stably expression, if integrated into the genome

of the APCs or provided as a stable extra-chromosomal element. Suitable genes used for modifying APCs include cytokine genes, adhesion molecules, interferon genes, chemokine genes and chemokine receptor genes and genes coding for heat shock proteins.

The vaccination composition of the invention may comprise additional molecules besides the nucleotide sequence and APCs. Such additional molecules include molecules that enhance or suppress the immune response of the subject, increase antigen presentation of APCs, activate the APCs and/or enhance the immune function of APCs. Included are cytokines, adhesion molecules, heat shock proteins, chemokines, such as interleukin-1 (IL-1), IL-2, IL-4, IL-6, IL-12, tumor necrosis factor α (TNF α), granulocyte-colony stimulating factor (G-CSF), macrophage-colony stimulating factor (M-CSF), granulocyte-macrophage-colony stimulating factor (GM-CSF), interferon γ (IFN γ), heat shock protein (hsp) 70, hsp90, gp96, CD40 ligand and B7, and adjuvants.

The present invention also refers to a method of treating and/or preventing a disorder or disease by administering the vaccine composition of the invention to a subject, preferably mammalian subject and more preferably human subject, in need thereof. In such a case, the antigenic molecule encoded by the nucleotide sequence is an antigenic molecule associated with the disease. For example, for an infectious disease, the nucleotide sequence preferably encodes a protein or peptide originating from the infectious microorganism, such as a viral, bacterial, fungi, protozoa or parasitic peptide or protein. Administration (injection) of the nucleotide sequence encoding the protein/peptide of the infectious microorganism together with modified APCs into a subject, preferably human subject, may elevate a specific immune response against the antigenic protein/peptide.

Several types of cancer are characterized by chromosome translocations in the cancer cells of the patient. Such translocations could result in connecting two or more coding sequences, or portions thereof, substantially

giving hybrid proteins or fusion polypeptides. Such a resulting hybrid protein can be recognized as non-self by the immune system and, thus, could have antigenic properties. A typical example is the e1a2 fusion protein associated with some types of leukemia.

In addition, many cancers are characterized by abnormal expression of certain genes, thus accumulating abnormal amounts of the corresponding proteins. Also such proteins, expressed in above-normal levels, can be used as antigens associated with a certain cancer type and therefore their associated gene sequences could be included in the nucleotide sequence of the vaccine composition of the invention.

The present invention also refers to a method of producing a vaccine composition. The method comprises identifying and isolating a nucleotide sequence encoding an antigenic molecule associated with a disease or disorder to be treated or prevented by the vaccine composition. In a preferred embodiment, the identified and isolated nucleotide sequence is cloned into a suitable vector, preferably a plasmid DNA vector. The resulting antigen encoding vector is then propagated, e.g. in host cells, *in vitro* and/or *in vivo*. APCs are isolated, preferably from the subject that subsequently will receive the vaccine composition. The APCs are preferably genetically modified by one or several genes encoding immune co-stimulatory molecules, which could increase antigen presentation of the APCs, activate the APCs and/or enhance the immune function of APCs. Finally, the antigen encoding nucleotide sequence (vector) and APCs are mixed completing the method and ending in a preferred embodiment of a vaccine composition of the invention. The final vaccine composition may optionally be provided with additional substances, including at least one of cytokines, adhesion molecules, chemokines, heat shock proteins and adjuvants.

The invention offers the following advantages:

- Superior efficiency compared to prior art DNA vaccine compositions;

- Eliminates the risk of introducing not fully inactivated pathogens as compared with vaccine compositions based on attenuated or inactivated pathogens;
- Vaccine could comprise xenogenic nucleotide sequences for breaking the tolerance of self-antigen and induce immune response against the self-antigen in a subject;
- Usable for treating and/or preventing a wide range of diseases and disorders by simply exchanging the antigen encoding nucleotide; and
- Allows introduction of immune co-stimulatory molecules by genetically modifying the antigen presenting cells of the vaccine composition.

Other advantages offered by the present invention will be appreciated upon reading of the below description of the embodiments of the invention.

SHORT DESCRIPTION OF THE DRAWINGS

The invention together with further objects and advantages thereof, may best be understood by making reference to the following description taken together with the accompanying drawings, in which:

Fig. 1 is a flow diagram illustrating a method of producing a vaccine composition according to the present invention;

Fig. 2 is a flow diagram of one of the steps of the vaccine producing method of Fig. 1;

Fig. 3 is a flow diagram of one of the steps of the vaccine producing method of Fig. 1;

Fig. 4 is a flow diagram of an additional step of the vaccine producing method of Fig. 1;

Fig. 5 illustrates CD40 ligand expression following successional gene-modifications of dendritic cells;

Fig. 6A illustrates expression of immune-response stimulatory molecules for parental BM185wt cells;

Fig. 6B illustrates expression of immune-response stimulatory molecules for D2SC/wt and gene-modified D2SC/CD40L cells;

Fig. 7A illustrates the effect of D2SC/wt and D2SC/CD40L cells in inducing allogenic T cell proliferation;

Fig. 7B illustrates the effect of D2SC/wt and D2SC/CD40L cells in inducing autologous T cell proliferation;

Fig. 8 schematically illustrates a portion of the wt pVAX-1 vector and a portion of the pVAX-e1a2 vector comprising a minigene sequence spanning the fusion region of e1a2. In addition the figure illustrates detection of the protein product of the minigene sequence by *in vitro* transcription-coupled translation assay of the plasmid vectors pVAX-1 and pVAX-e1a2;

Fig. 9 illustrates the percentage of tumor free mice after administration of different vaccine compositions to mice with pre-existing bcr/abl positive tumors followed by rechallenge with live parental tumor cells;

Fig. 10 is a comparison of the vaccine composition of the present invention with other vaccine compositions in treating mice with pre-existing bcr/abl positive tumors;

Fig. 11A illustrates the induction of tumor-specific CTL response directed against parental BM185 cells for different vaccine compositions;

Fig. 11B illustrates the induction of tumor-specific CTL response directed against TAP deficient RMA-S cells pulsed with e1a2 peptide for different vaccine compositions; and

Fig. 12 illustrates the percentage of e1a2 specific T cells following different vaccination strategies.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which the present invention belongs. The following references provide a general definition of many of the terms used in this invention [12-17]. For clarity of the invention, the following definitions are used herein.

"Nucleotide sequence" or "nucleic acid sequence" refers to a polymer composed of nucleotide units (ribonucleotides, deoxyribonucleotides, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof) linked via phosphodiester bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. It will be understood that when a nucleotide sequence is represented by a DNA sequence (i.e., A, T, G, C), this also includes an RNA sequence (i.e., A, U, G, C) in which "U" replaces "T". The term nucleotide sequence includes, unless otherwise specified, double stranded and single stranded DNA, cDNA and RNA. Also hybrids such as DNA-RNA hybrids are included in the term. Reference to a nucleotide sequence or nucleic acid sequence can also include modified bases known to the person skilled in the art.

The term "encoding " or "coding for" refers to the inherent property of a nucleotide sequence, such as a gene, a cDNA or an mRNA, to serve as templates for synthesis of other molecules in biological processes having either a defined sequence of nucleotides (i.e. rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting

therefrom. Thus a gene encodes a protein if transcription and translation of mRNA produced by that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence, and the noncoding strand, used as the template for transcription of a gene or cDNA can be referred to as encoding the protein or other product of that gene or cDNA.

The term "coding sequence" and "coding nucleotide sequence" refers to a nucleotide sequence with the properties of being able to be transcribed into either a defined sequence of nucleotides (tRNA, rRNA and mRNA) and, in the case of mRNA transcription, being further translated into a polypeptide. The coding sequence may be a gene, a cDNA or a recombinant nucleic acid.

"Promoter" is the minimal nucleotide sequence required to direct transcription. The promoter could be a constitutive promoter or could include elements that render the promoter-depending gene expression cell-type or tissue specifically controllable or inducible by external signals or agents.

"Immune system" refers to the molecules, cells, tissues and organs that collectively function to provide immunity or protection against foreign organisms.

"Antigen" refers to a molecule that binds to an antibody or a T cell receptor (TCR). Antigens that bind to antibodies include all classes of molecules. TCRs only bind peptide fragments of proteins associated with major histocompatibilty complex (MHC).

The term "antigen presenting cell (APC)" refers to a cell that displays peptide fragments of a protein antigen in association with MHC molecules on their cell surface. APC activates antigen specific T cells and express co-stimulatory molecules to optimally activate T cells. In particular, APC refers to

professional APC having MHC class II molecules, including dendritic cells (DCs), monocytes, macrophages and B cells.

The expression "modified APC" refers to an APC cell acquiring genetic information received by manipulation, including viral and non-viral vector manipulation. As a result of the (genetic) manipulation, the genetic material is incorporated in the ADC, where the material preferably is integrated within the cell's chromosomes but could also be replicated extra-chromosomally.

According to an aspect of the present invention there is provided a vaccine composition comprising of an isolated or substantially purified nucleotide sequence or nucleic acid sequence coding for an antigen molecule and gene-modified antigen presenting cells (APCs). The vaccine composition is preferably provided as an intermixture of the nucleotide sequence and the APCs, i.e. the nucleotide sequence and the APCs are preferably pre-mixed prior administration to a subject. This novel vaccine composition obtains increased immune response and superior therapeutic effect compared to the prior art DNA vaccines, in particular in eliminating pre-existing cancer cells and protect the animal from rechallenge of tumor cells.

The nucleotide sequence according to the present invention could be naked DNA or RNA administered together, preferably as a mixture, with the gene-modified APCs. If provided as a RNA sequence, the nucleotide sequence includes motifs allowing translation thereof in cells of a subject receiving an administration of the vaccine composition. Likewise, if provided as DNA, the nucleotide sequence includes motifs, such as promoter and possibly enhancers, allowing transcription of the antigen encoding (DNA) sequence.

The nucleotide-based vaccine of the invention may be a univalent or multivalent vaccine. In the former univalent case, the nucleotide sequence encodes one antigen, where in the multivalent case the sequence encodes two or more different antigens. Thus, for a multivalent vaccine several antigens become expressed when administered in a subject, essentially

resulting in activation of the antigen-specific T cells recognizing the different antigens. It is also possible to include several copy of one antigen encoding sequence, e.g. provided in duplicate, triplicate, etc. The nucleotide sequence of the vaccine composition could also include other immune co-stimulating or effecting sequences, such as gene sequences coding for (protein or peptide) molecules having an immune response co-stimulating effect. Also co-stimulating DNA sequences, such as unmethylated cytidine phosphate guanosine motifs, can be included in the nucleotide sequence, which is discussed in more detail below.

However, the antigen encoding sequence is preferably included in a vector under transcriptional control of a promoter, e.g. included in an expression cassette of an expression vector with an expression control sequence, i.e. promoter, controlling transcription of the associated antigen encoding nucleotide (gene) sequence. When injected into a subject, the vector is taken up by cells, which transcribes the nucleotide sequence resulting in a RNA antigen or transcribes and translates the sequence resulting a protein/polypeptide antigen, which is discussed in more detail below. Furthermore, the vector or expression control sequence of the vector preferably comprises other regulatory sequences required for efficient transcription/translation of associated antigen encoding nucleotide sequence, including, but not limited to, polyadenylation sequence, transcription sequence and enhancers. The promoter, associated with and controlling expression of the antigen encoding sequence, is chosen so that it is active in the subject to be vaccinated, or inducible active or active in some of the cells of the subject. Examples of suitable promoters for vaccination of human subjects include virus promoters, e.g. cytlomegavirus (CMV) promoter.

An example of a vector that could be used according to the invention is liposomes. Liposomes are vesicles composed of phospholipid bilayer membranes that can enclose various substances, including DNA. Various cationic lipid formulations have been described for DNA delivery to cells.

Although these agents are effective *in vitro* in a number of cell lines, major problems have been observed when they are administered *in vivo* [18]. Liposomes are cleared from the circulation rapidly after intravenous injection. As a result, their use has been largely restricted to intralesional injection. Insertion of polyethylene glycol derivatives into the lipid membrane or pegylation of the liposomes can increase the circulation half-life of liposomes after intravenous administration.

Another class of synthetic vectors that have been actively studied is cationic polymers. The general principle is based on complex formation between positively charged polymers and negatively charged DNA molecules. Compared with cationic lipids, cationic polymers are more efficient in condensing DNA. Examples of polymers evaluated for gene delivery are poly-L-lysine, polyethylenimine (PEI), polyglucosamines and peptoids. For example, PEI has been shown to protect complexed DNA from degradation within endosomes and it also provides a means of promoting DNA release from the endosomal compartment and its subsequent translocation to the nucleus [19].

A presently preferred vector according to the invention is a DNA plasmid vector incorporating the antigen encoding sequence. Noteworthy, use of bacterial DNA plasmid vectors according to the present invention, either as naked DNA or embedded in liposomes or cationic polymers, offers a further advantage. Bacterial plasmid vectors contain immunostimulatory nucleotide sequences, namely unmethylated cytidine phosphate guanosine motifs (CpG, 5'-purine-purine-CG-pyrimidine-pyrimidine-3'), capable of causing maturation and activation of APCs. In addition, the CpG motif can elicit NK-cell activity, B cell proliferation and immunoglobuline production and cytokine secretion. Thus, in addition to allowing delivery and expression of an antigen of the invention in a subject, a bacterial plasmid vector stimulates the immune response of the subject.

Alternatively, viral systems can be used for delivering and subsequently expression of the antigen encoding nucleotide sequence of the invention. Viral systems generally achieve high efficiencies of gene transfer, in many cases also in non-dividing cells. Viruses are attractive vehicles for nucleotide sequence delivery since they have evolved specific and efficient means of entering human cells and expressing their genes. The main challenge for viral vector development lies in keeping the targeting efficiency of viruses, while abrogating their ability to cause infection and disease. This is achieved by modifying the viral genome to remove sequences required for viral replication and pathogenicity. The removed viral coding sequences can be replaced with exogenous genes, i.e. the antigen encoding nucleotide sequence. Such genetically engineered viruses theoretically retain wild-type viral cellular tropism and ensure transgene expression in the target cell population without causing ongoing infection. Gene delivery using replication defective virus vectors is referred to as transduction. To date there are four types of viral vectors in clinical trials: retroviruses, adenoviruses, herpes simplex virus and adeno-associated viruses. Other viruses that are under investigation include pox virus, reovirus, Newcastle disease virus, alphaviruses and vesicular stomatitis virus, which also may be employed as vectors according to the invention.

The nucleotide sequence of vaccine of the present invention encodes an antigenic molecule, RNA or preferably peptide/protein, that when expressed in a subject elicits the immune response. The antigen is preferably an immunogenic molecule, an immunogenic fragment of a molecule, such as an immunogenic protein, peptide or RNA molecule or fragment thereof. Furthermore, depending on the actual type of vaccination composition, the antigen encoded by the nucleotide sequence could be a tumor-associated or tumor antigenic molecule, bacterial, parasitic, viral, fungi or protozoa protein/peptide or RNA molecule, an allergen or a self protein/peptide, or antigenic fragment thereof.

Once injected into a subject, the nucleotide sequence or vector is taken up by the subject's cells and is translocated to cell nuclei (for a DNA nucleotide sequence and vector), where the antigen is expressed. For a preferred DNA plasmid vector, once the plasmid is taken up by the host cells, it will be translocated into cell nuclei and persist as circular non-replicating episomes without intergrating into the host genome. In either way, the produced antigen molecule is processed in the cytosol into peptides by proteasomes. Membrane-associated transporters of antigenic peptide (TAP) move these digested peptides into the endoplasmic reticulum where they interact with major histocompatibility complex (MHC) class I molecules. MHC is a heterodimeric membrane protein that serves as a peptide display molecule for recognition by T cells. Two distinct types of MHC molecules exist. MHC class I molecules are presented on the surface of nearly all nucleated cells, and bind to peptides derived from cytosolic proteins and recognized by CD8+ T cells. In contrast, MHC class II molecules are restricted largely to professional antigen presenting cells (APCs), bind peptides derived from endocytosed proteins and present antigenic peptides to CD4+ T cells. The MHC I with bound peptide is then transported and become anchored in the cell membrane. There the MHC class 1-peptide complex is displayed and recognized by CD8+ T cells, which become activated. Once activated, the CD8+ T cells acquire cytotoxic functions and can specifically lyse cells expressing the target antigen.

Furthermore, after vaccine administration, professional antigen presenting cells (APCs) either directly acquire antigen or take up antigens released from other transfected cells. Lysis of cells transfected with vector or nucleotide sequence of the invention leads to release of encoded antigen, which is taken up by APCs. As it is internalized into lysosomes, the antigen is proteolytically degraded into peptides associating with MHC class II molecules. The MHC class II-peptide complexes travel to the cell surface of APCs, where they are recognized by CD4+ T cells. Activated tumor-specific CD4+ cells not only secrete cytokines and provide help for the induction of specific CD8+ cytotoxic T lymphocytes (CTL), but may also play important roles in

activating macrophages and eosinophils to produce nitric oxide and superoxides that participate in the destruction of cells expressing the target antigen. CD4+ T cells activated B cell to produce antibodies and induce humoral immune responses. Thus, immunization with a vaccine composition according the invention induces both antigen-specific cellular as well as humoral immune responses.

Furthermore, since the nucleotide sequence with the antigen of the invention is preferably pre-mixed with APCs, some the APCs will take up the nucleotide sequence and start expressing the antigen prior administration to a subject. Thus, the APCs will be activated and presents antigens to T cells directly after administration, thereby obtaining a faster and more efficient immune response.

The APCs of the present invention are cells adapted for presentation of antigens to T cells and/or B cells of the immune system. The APCs preferably have a high content of MHC class I and class II molecules allowing efficient antigen presentation. Furthermore, the APCs preferably also express other cell-surface molecules including, without limitation, high levels of adhesion molecules and co-stimulatory molecules, which are required for the activation of T cells. In addition, the APCs of the present invention preferably express chemokins and chemokine receptors.

In an embodiment, the APCs of the vaccine of the present invention can be obtained from a subject, preferably the same subject to whom the vaccination composition is to be administered, i.e. autologous APCs are used. Alternatively, non-autologous APCs can be used. The non-autologous APCs can be syngeneic, i.e. from an identical twin of the subject, or allogeneic, i.e. an individual who shares at least one common MHC allele with the subject.

In another embodiment, the APCs can optionally be enriched or purified and/or expanded *ex vivo* by methods well known in the art. For example,

without limitation, the APCs are obtained by production *in vitro* from stem and progenitor cells from human peripheral blood or bone marrow as described in [20].

Preferred APCs of the invention are professional APCs, including DCs, macrophages, monocytes and B cells, or a mixture of at least two of DCs, macrophages, monocytes and B cells. Also Langherhans cells, vascular endothelial cells and/or various epithelial and mesenchymal cells could be used as APCs according to the invention.

DCs are highly specialized APCs of hematopoietic origin that process unique immunostimulatory properties and function as the principle activators of quiescent T cells and play crucial roles in the initiation and regulation of immune responses [21]. These DCs express a unique repertoire of cell-surface molecules including high levels of MHC class I and II, adhesion molecules and co-stimulatory molecules (CD40, CD54 and CD86), all of which are required in the activation of T cells. DCs are motile cells with elaborate cytoplasmic processes and a unique veiled morphology.

Macrophages must be activated by phagocytosing microorganisms before expressing MHC class II complex or the co-stimulatory B7 molecule.

B cells constitutely express the MHC class II complex but first have to be activated to express co-stimulatory molecules.

In a preferred embodiment of the present invention the APCs of the vaccination composition are (genetically) modified APCs. In such a case, the APCs are modified to express molecules that effect, i.e. enhance or suppress, the immune response, increase antigen presentation, activate the APCs and/or enhance the immune function of APCs. Thus, genetic material has been introduced into the APCs for transiently expression from an episomal location or stably expression if integrated into the genome of the APCs or provided as a stable extra-chromosomal element. Suitable genes used for

modifying APCs include cytokine genes, adhesion molecules, interferon genes, chemokine genes, chemokine receptor genes and genes encoding different immune co-stimulating molecules, ligands and receptors.

Suitable gene delivery protocols for modifying APCs include, without limitation, viral and non-viral methods. Viral vectors include, without limitation, retrovirus, adenovirus, adeno-associated virus, vaccina virus, herpes simplex virus and lentvirus. Non-viral delivery of gene into DCs includes, without limitation, plasmid DNA transfection, liposomes and microinjection.

The antigen encoding nucleotide sequence and (genetically modified) APCs of the vaccine composition are provided and administered in an isotonic, preferably buffered solution, suitable for use in administration to a subject, preferably a human subject. An example of such a solution is a PBS solution.

The vaccination solution of the invention may comprise additional molecules besides the sequence and APCs. Such additional molecules could include molecules that effect (enhance or suppress) the immune response of a subject, increase antigen presentation of APCs, activate the APCs and/or enhance the immune function of APCs. Included are cytokines, adhesion molecules, heat shock proteins and chemokines, such as interleukin-1 (IL-1), IL-2, IL-4, IL-6, IL-12, tumor necrosis factor α (TNF α), granulocyte-colony stimulating factor (G-CSF), macrophage-colony stimulating factor (M-CSF), granulocyte-macrophage-colony stimulating factor (GM-CSF), interferon γ (IFN γ), CD40 ligand, CD40, B7, chemokine, chemokine receptor, heat shock protein (hsp) 70, hsp90 and gp96.

The solution may also comprise adjuvants enhancing or suppressing the immune response, increasing the antigen presentation, redirecting the vaccine to the immune system and/or facilitating DNA entry into cells. Adjuvants include, without limitation, mineral salt adjuvants or mineral salt

gel adjuvants, particulate adjuvants, microparticulate adjuvants, mucosal adjuvants and immunostimulatory adjuvants. Examples of adjuvants include aluminium hydroxide, aluminium phosphate gel, Freund's complete adjuvant, Freund's incomplete adjuvant, squalene or squalene oil-in-water adjuvant formulations, biodegradable and biocompatible polyesters, polymerized liposomes, triterpenoid glycosides or saponins, N-acetyl-muramyl-L-threonyl-D-isoglutamin, LPS and monophosphoryl lipid A.

Another aspect of the invention is use of a vaccine composition for producing an immune response in a subject. In such a case, a vaccine composition, which comprises a nucleotide sequence encoding an antigenic molecule against which an immune response is desired to be induced, and gene-modified APCs, is administered to the subject, preferably a mammalian subject and more preferably a human subject.

A further aspect of the invention is use of a vaccine composition for treating and/or preventing a disease in a subject. In such a case, a vaccine composition, which comprises a nucleotide sequence encoding a molecule that displays antigenicity of an agent that causes the disease, and gene-modified APCs, is administered to a subject, preferably a mammalian subject and more preferably a human subject, in need thereof.

The disease to be vaccinated against or to be treated may be an infectious disease, cancer, allergy and/or diabetes. In such a case, the nucleotide sequence in the vector of the vaccine composition encodes an antigene molecule (RNA and/or protein/peptide) associated with the disease. Once expressed (transcribed and possibly also translated) in a subject, the molecule elicits an immune response.

Infectious diseases to be treated or prevented by usage of a vaccine composition of the invention are caused by infectious agents including, but not limited to, viruses, bacteria, fungi, protozoa and parasites. In either way, the antigen encoding nucleotide sequence of the vaccine composition is

coding for an antigenic molecule associated with the pathogenic microorganism causing the disease. Furthermore, this antigenic molecule is preferably recognized as non-self by the immune system of the subject to be vaccinated.

Viral diseases that may be treated or prevented by the vaccine of the invention include those caused by adenovirus, arbovirus, coxsackie virus, cytomegalovirus, echinovirus, echovirus, hantavirus, hepatitis A virus, hepatitis B virus, hepatitis C virus, herpes simplex I virus, herpes simplex II virus, human immunodeficiency virus type I, human immunodeficiency virus type II (HIV env protein could be used as antigenic molecule), influenza (NP antigen could be used as antigenic molecule), measles virus, mumps virus, papilloma virus, papova virus, polio virus, respiratory syncytial virus, rhinovirus, rinderpest, rotavirus, rubella virus and varicella.

Examples of infectious diseases caused by legionella, mycobacteria (hsp65 antigen can be used as antigenic molecule of *Mycobacterium tuberculosis*), mycoplasma, neisseria and rickettsia bacteria may be prevented and/or treated by the vaccine composition of the invention.

Protozoa caused diseases that may be treated by the vaccine composition of the invention are diseases caused by kokzidioa, leishmania and trypanosoma. Whereas corresponding parasitic diseases could be caused by chlamydia, rickettsia and Leishmania major murine infection (antigenic molecule could be the LACK antigen).

The vaccine composition of the invention is also well adapted for use in preventing and/or treating different types or forms of cancer. The antigen encoding nucleotide sequence of the vaccine composition then encodes an antigenic molecule that is associated with the particular cancer type. Several cancers, such as chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL), are characterized by gene or chromosome translocations in the cancer cells of the patient. Such translocations could result in

connecting two or more coding sequences, or portions thereof, substantially giving hybrid or fusion proteins or polypeptides. Such a resulting hybrid protein can be recognized as non-self by the patient's immune system and, thus, could have antigenic properties.

In addition, many cancers are characterized by abnormal expression of certain genes, thus accumulating abnormal amounts of the corresponding proteins. Also such self-proteins, expressed in above-normal levels, can be used as antigens associated with a certain cancer type. However, these self-antigens are expressed not only by tumor cells but also by normal host tissue. In order to induce an effective antitumor immune response, the vaccination must overcome the immunological tolerance of the self-antigens. One alternative approach according to the invention is to use a xenogenic source, e.g. a DNA plasmid vector, in which the tumor-associated antigen encoding sequence is provided, to break the tolerance of the corresponding self-antigen and to induce tumor immunity. In addition, the immune system contains autoreactive T and B cells that are not necessarily deleted from the immune repertoire during development. The autoreactive lymphocytes may be triggered by cross-reactivity between species. Cross-reactive immunity to a mouse self-antigen can be induced by immune recognition of the corresponding human protein following xenogenic DNA immunization. Thus, for obtaining an immune response in a subject, a nucleotide sequence encoding a selft-antigen is preferably provided in a xenogenic vector, e.g. a xenogenic DNA plasmid vector that comprises xenogenic sequences and preferably encodes xenogenic proteins and peptides in order to break the tolerance of the self-antigen.

Non-limiting examples of tumor-specific or tumor-associated antigens, the coding sequence of which may be used in the vaccine composition of the invention, include KS 1/4 pan-carcinoma antigen [22], ovarian carcinoma antigen (CA125) [23], prostatic acid phosphate, prostate specific antigen [24, 25], melanoma-associated antigen p97 [26], melanoma antigen gp75 [27], high molecular weight melanoma antigen [28], the MAGE family of antigens

[29, 30], T cell receptor γ chain alternate reading frame protein (TARP) antigen [31, 32], prostate specific membrane antigen and e1a2 fusion protein antigen [33].

Cancer vaccines for treatment of melanoma, pancreatic carcinoma, breast cancer, prostate cancer are presently used in clinical trials. The vaccine composition of the invention could then be used for these cancer types with superior results compared to the present prior art cancer vaccines. Further non-limiting examples of cancers that may be treated and/or prevented by usage of the vaccine composition of the present invention are the following types of cancer: human sarcomas and carcinomas, e.g. fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, enotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung cacinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma and retinomblastoma, leukemias, e.g. acute lymphocytic leukemia (ALL), and acute myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erytholeukemia), chronic leukemias (chronic myelocytic leukemia, chronic granulocytic leukemia and chronic lymphocytic leukemia), polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenström's macroglobulinemia and heavy chain disease.

The vaccine composition of the invention can be employed for eliminating pre-existing tumors or pathogens, thus basically being used for treating cancer or an infectious disease. However, the vaccine composition can also or alternatively be used to protect a subject, preferably a mammalian subject and more preferably a human subject, from a challenge or relapse with tumor cells or the pathogenic infectious agent (microorganism). In such a case, the vaccine is basically used for preventing cancer or an infectious disease, i.e. having prophylactic properties.

Different allergies may be treated by the present invention by using different antigen encoding nucleotide sequences in the vector. The sequence to use depends on the particular type of allergy, and can be selected by the person skilled in the art. For example, for peanut allergy, the vector could include a nucleotide sequence encoding the Arah 2 antigen.

For type I diabetes, the vaccine composition of the present invention can, without limitation, include a nucleotide sequence encoding insulin, or a portion thereof.

The vaccine composition of the present invention may be administered to a subject, preferably mammalian subject and more preferably human subject, using suitable vaccination routs including, without limitation, subcutaneously, intramuscularly, intra-arterially, intravenously, intravascularly, orally, intradermally, intraperitoneually, directly injection into lymph notes and intratumor injection. The antigen encoding nucleotide sequence and the gene-modified APCs are preferably intermixed prior the administration, i.e. administrating a mixture of the two components of the vaccine composition of the present invention.

The vaccine may be administered by any conventional means, without limitation, including syringe, trocar, catheter, or like.

The dosage to be administered depends to a large extent on the condition and size of subject to be treated/vaccinated as well as the amount of vaccine composition administered, frequency of administration, administration route, type of therapy, i.e. treatment and/or prevention, and type of disease to be treated or vaccinated against. Regimens for continuing therapy, including site, dose and frequency may be guided by the initial response of the subject and clinical judgement. However, for treatment of cancer, preferably at least three repeated vaccinations are given. The amount of vaccine composition to be used can be determined by dose-response experiments conducted in animals by methods well known in the art.

Yet another aspect of the invention is a kit, provided for usage in the vaccination methods of the present invention. In a first embodiment a kit comprises a container including a mixture of a nucleotide sequence encoding an antigenic molecule and APCs, preferably genetically-modified APCs. The antigenic molecule is preferably associated with a disease, e.g. infectious disease or cancer, to be treated or prevented by administering the contents of the kit to a subject, preferably mammalian subject and more preferably human subject.

In another embodiment a kit comprises a first container, including a preparation of a nucleotide sequence that codes for an antigenic molecule, and a second container, including a preparation of genetically-modified APCs. Prior administration to a subject, the content of the first and second container are preferably intermixed, e.g. by adding the content of the first container to the second container, by adding the content of the second container to the first container or by adding the content of the first and second container, respectively, to a third provided mixing container. The mixed composition is then administered to a subject, preferably mammalian subject and more preferably human subject.

The kits of the invention comprise a vaccine composition in amount effective to treat and/or prevent a disease or disorder, e.g. infectious disease, cancer,

allergy or diabetes. At least one of the containers of the kit, preferably the container comprising gene-modified APCs, may include additional substances and adjuvants effecting the immune response of a subject, increasing antigen presentation of APCs, activating the APCs and/or enhancing the immune function of APCs. Suitable additional substances and adjuvants may be selected from the cytokines, adhesion molecules, chemokines, heat shock proteins and adjuvants discussed in the foregoing.

A further aspect of the invention is a method of producing a vaccine composition. Referring to the flow diagram of Fig. 1, in step S1 a nucleotide sequence encoding an antigenic molecule (against which an immune response is desired to be induced) is provided. Gene-modified APCs, e.g. DCs, macrophages, monocytes and/or B cells are provided in step S2. In step S3, the antigen encoding sequence and APCs are mixed completing the method and ending in the vaccine composition of the invention.

Fig. 2 illustrates a preferred embodiment of the nucleotide sequence-providing step S1 of Fig. 1 in more detail. In the optional step S11 the nucleotide sequence encoding the antigenic molecule associated with a disease or disorder to be treated or prevented by the vaccine composition is identified and isolated. Any methods known in the art, including chemical synthesis of DNA sequences and PCR (polymerase chain reaction), can be employed to obtain the relevant nucleotide sequence. Then in step S12 the identified and isolated nucleotide sequence is preferably cloned into a suitable vector. The vector is selected for being adapted for introduction into a subject and for, once introduced into the subject, enabling expression of the nucleotide sequence, subsequently resulting in the desired antigen molecule. The obtained antigen encoding vector is then propagated in step S13, e.g. in host cells, *in vitro*, etc.

The APC providing step S2 of Fig. 1 is illustrated in more detail in Fig. 3. In step S21, APCs are isolated, preferably from the subject that subsequently will receive the vaccine composition or from another source discussed in the

foregoing. The APCs are (genetically) modified in step S22. In such a case, one or several genes, e.g. CD40 ligand gene, coding for molecules that increase antigen presentation of the APCs, activate the APCs and/or enhance the immune function of APCs, are introduced, either as extra-chromosomal elements or incorporated in the genome of the APCs, into the antigen presenting cells.

In the further step S4 of Fig. 4 additional substances are added to the vector-APCs mixture. Such substances could include at least one of cytokines, adhesion molecules, chemokines, heat shock proteins and adjuvants discussed in the foregoing, generally effecting the immune response of a subject receiving the vaccine composition and/or enhancing the immune function of APCs.

In the following the vaccine composition of present invention will be exemplified with reference to a vaccine against a type of acute leukemia. Thus, the molecule encoded by the nucleotide sequence is in the following a protein associated with that cancer type. However, as the person skilled in the art understands the invention is not limited to this particular example of disease and antigen but can be used to treat and/or prevent any of the diseases and disorders discussed above.

Today, the mortality of acute leukemia is high. A usual form of acute leukemia, acute lymphoblastic leukemia (ALL) progresses quickly and can occur in both children and adults. ALL is the most common malignancy in children and accounts for approximately 80 % of childhood leukemia. Patients with ALL have progressive infiltration of immature hematopoietic cells in bone marrow and peripheral blood. These cells can also accumulate in the lymph tissues and make them swell. Lymphocytes may crowd out other blood cells in the blood and bone marrow. If the bone marrow cannot make enough red blood cells to carry oxygen, then anemia may develop. If the bone marrow cannot make enough platelets to make the blood clot normally, bleeding or bruising may develop more easily. The leukemic

lymphocytes can also invade other organs including the spinal cord and the brain.

The Philadelphia (Ph^1) chromosome translocation t(9;22) is regularly found in malignant cells of patients with chronic myeloid leukemia (CML) and some patients with acute lymphoblastic leukemia (ALL). Philadelphia translocation involves the c-abl oncogene moved from chromosome 9 into the breakpoint cluster region (bcr) within the bcr gene on chromosome 22, resulting in generation of a chimeric *bcr/abl* gene. The fused *bcr/abl* gene encodes an 8.5 kb chimeric mRNA which is translated into a 210-kDa or 190-kDa protein. This bcr/abl protein exhibits tyrosine kinase activity and is uniquely present in the leukemia cells. In Ph^1 positive ALL, the predominant breakpoint is at the ela2 site, resulting in expression of a p190-e1a2 bcr/abl protein. The e1a2 fusion protein is a unique tumor antigen for ALL that is not found in normal cells. Therefore, the unique amino acid sequence encompassing the ela2 breakpoints can be considered as truly a tumor specific antigen.

Current conventional treatments using a combination of chemotherapy and bone marrow transplantation cure approximately 65 % of affected children [34]. The age of the patient at diagnosis has strong prognostic significance, reflecting the different underlying biology of ALL in different age groups. In adults with ALL, the disease is curable in only 20-35 % of the patients although much progress has been made.

Based on the molecular and biological characteristics, leukemia cells are classified into subgroups. B-lineage ALL is defined by the expression of CD19, HLA-DR, CD10 (cALLa), and other B cell associated antigens and represents 80-85 % of childhood ALL. There are three major subtypes of B-lineage ALL: early pre-B (no surface or cytoplasmic immunoglobulin), pre-B (presence of cytoplasmic immunoglobulin), and B cell (presence of surface immunoglobulin). Approximately three-quarters of patients with B-precursor ALL have the early pre-B phenotype and have the best prognosis.

EXAMPLE

The vaccine composition of present invention was compared with a variety of other approaches including vaccinations with peptides derived from the e1a2 fusion protein. Amino acids sequences of ALL-specific minor e1a2 fusion protein were used for screening by binding to mouse MHC-I antigen (H-2K^d) (HLA Binding Prediction, derived from Dr. Kenneth Parker's Research, http://bimas.dcrt.nih.gov/molbio). A nine amino acid sequence, AFHGDAEAL, which spans the junction-region of e1a2 fusion protein, see Fig. 8 and SEQ ID NO: 5, was shown to have high score binding to mouse H-2K^d. Thus, the peptides covering the e1a2 mini-protein was synthesized by standard methods and purified by high pressure liquid chromatography (HPLC). Peptides used in the experiments were the high score binding peptide (e1a2 peptide, AFHGDAEAL, see SEQ ID NO: 5) and low score binding peptide (peptide 8, HGDAEALQ, see SEQ ID NO: 6). Peptide K (ATGFKQSSK, see SEQ ID NO: 7) that does not bind to H-2K^d was used as control peptide.

Cell line

A20 (H-2^d) is a B cell lymphoma cell line derived from BALB/c mice used as CTL target in the present invention study. A20 cells express B220, MHC-I, MHC-II and CD19 molecules.

Transgenic mice expressing the human *bcr/abl* fusion gene (e1a2 gene) were established recently [35]. These mice consistently develop leukemia of B-cell origin (BM185) after a short period of latency. The tumor cells (BM185wt, H-2^d) is a murine acute leukemia cell line (pre-B ALL) and was established from bone marrow cells of BALB/c mice transduced with retroviral vector encoding a 185-kDa bcr/abl oncoprotein [35]. In addition, BM185wt cells express B220, CD19 and MHC-I molecules on their cell surface.

Cell culture

DCs were cultured in IMDM medium supplemented with 10 % heat inactivated fetal calf serum (FCS) (GibcoBRL, Life Technologies Ltd., Scotland, UK), 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin, 10 mM hepes and 5×10^{-5} M 2-mercaptoethanol. Cells were incubated at 37 °C in a humidified atmosphere of 7 % CO_2.

Tumor cells were cultured in RPMI-1640 medium (ICN Biomedicals, Inc. Costa Mesa, CA) supplemented with 10 % heat inactivated FCS (GibcoBRL, Life Technologies Ltd. Scotland, UK), 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin, 10 mM hepes, 0.1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol and 10 mM non-essential amino acids. Cells were incubated at 37 °C in a humidified atmosphere of 5 % CO_2.

Statistical analysis

All animal experiments were conducted with a minimum of five or ten animals per group. All studies were repeated at least three times with similar results. Survival differences were analyzed with the Chi-square test to interpret the significance of differences between experimental groups.

Genetically modification of dendritic cells

A gene transfer system based on retroviruses was developed. The retroviral provirus is manipulated so that all of the *gag*, *pol* and *env* genes are removed but the 3'- and 5'-LTRs are retained. The manipulated provirus is propagated as plasmid in *E. coli* and then the DNA is used to transfect a helper-free packaging cell line. Thus, the defective retrovirus vector is produced in the supernatant from the packaging cell line. In the present invention a Moloney murine leukemia virus vector (MoMLV) containing CD40 ligand gene (muCD40L) (kind gift of Dr. M. Brenner, Baylor College of Medicine, USA) was used to infect DCs.

The DCs (D2SC/wt, H-2^d) used in the vaccine of present invention was obtained by retroviral immortalization of DCs from BALB/c spleen [36] and

the DCs are further engineered to express CD40 ligand on their cell surface. The CD40 ligand gene is transferred into DCs using the muCD40L retroviral vector. Dendritic cells (D2SC/wt) were transduced by repeated spinoculation in the presence of polybrene (10 µg/ml, Sigma). In brief, 10^5 cells were suspended in 0.5 ml viral supernatant and polybrene. Cells and virus were co-centrifuged at 10,000 rpm, at room temperature for 60 min. After centrifugation, the supernatant was discarded and the cells were suspended in fresh medium and incubated at 37 °C in a humidified atmosphere of 7 % CO_2 for 24 h prior to a second round of infection. The transduction efficiency of DCs was markedly enhanced by repeated centrifugation. Several rounds of transductions were performed and the corresponding percentage of cells expressing CD40L is illustrated in Fig. 5.

After several rounds of transduction, approximately 70-80 % of DCs expressed CD40L transgene. The D2SC/CD40L cells were further sorted for the transgene expression and cryopreserved in large batches. The transgene expression remained stable even after repeated thawing-freezing procedures. After repeated transduction, the DCs express readily CD40 ligand on their cell surface, see bottom diagram of Fig. 5.

Cell culture supernatant of CD40 ligand transduced DCs and CTLs was harvested and evaluated for IL-12 production by ELISA according to the manufacturer's instructions (Cytoscreen™, immunoassay kit, Biosource Int., California, USA). Interestingly the transduced D2SC/CD40L cells produced non-detectable level of IL-12 in cell culture supernatant (data not shown).

<u>Immunophenotype characterization of tumor cells, DCs and DC40L gene-modified DCs</u>

Cells were incubated with mouse monoclonal antibodies (2×10^5 cells/0.5 µg mAbs, Pharmingen, San Diego, CA) against a panel of surface molecules. The following mAbs were used: CD40-FITC (fluorescein isothiocyanate conjugated mAB), CD40L-PE (R-phycoerythrin), I-A-FITC, H-2K^d-FITC, B7.1-FITC, B7.2-PE, CD11c-FITC, CD8α-PE, B-220-FTIC, B220-PE, Thy1.2-FITC,

Thy1.1-PE, IgG1-FITC, IgG2-PE. All monoclonal antibodies are rat anti-mouse except CD40L-PE, which is a hamster anti-mouse mAb. Phenotypic analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson).

The immunophenotype of tumor cells (BM185/wt) is illustrated in Fig. 6A. The corresponding immunophenotypes of parental D2SC/wt cells and CD40 ligand gene-modified dendritic cells (D2SC/CD40L) are compared and illustrated in Fig. 6B.

The tumor cells, BM185wt (H-2^d) express a human p185 e1a2 oncoprotein [35]. In addition, the tumor cells express B220, CD19 and MHC-I, I-A (see Fig. 6A) molecules on their cell surface. However, BM185wt cells lack expression of co-stimulatory molecules, such as B7, which is clearly seen from Fig. 6A.

The parental D2SC/wt cells represent an immature DC phenotype [37, 38] and express MHC-1, I-A, CD8α, CD11c, B7.1 and B7.2 on their cell surface, as is illustrated in Fig. 6B, but no CD40 ligands. In comparison, readily expression of CD40L was found in D2SC/CD40L cells. However the IL-12 secretion was not detected in D2SC/CD40L cell culture media. *In vitro* growth kinetics of D2SC/CD40L cells was similar to their parental cells (data not shown).

T cell activation

CD40 ligand is predominately expressed by activated T cells. Previous study of vaccination with neuroblastoma cells modified to express CD40L has shown delayed tumor growth and induction of protective anti-tumor immunity [39].

Stimulators (DCs and tumor cells) were irradiated (25 Gy, from ^{137}Cs) and added in graded doses to responder cells (spleenocytes or purified T cells isolated from BALB/c mice, 2×10^5 cells/well) in 96-well round-bottom

microtiter plates (Becton, Dickinson). Experiments were performed in triplicates in a final volume of 200 µl/well. Proliferation was measured by the uptake of [^{3}H]-thymidine (1 µCi/well) (Amersham International, Amersham, UK) added for 6 h on day 3 prior to harvesting onto glass fiber filter and subjected to scintillation counting (Beta counter, Pharmacia).

CD40 ligand gene-modification elevates DCs capacity to stimulate T cells. Co-culture of D2SC/CD40L cells with allogenic or autologous T cells induced a strong T cell proliferation, as is shown in Figs. 7A and 7B. In contrast, the parental D2SC/wt cells induce low-levels of T cell proliferation.

Thus, a vaccine composition of present invention preferably comprises gene-modified, e.g. CD40L gene-modified, DCs eliciting both superior allogenic and autologous T cell proliferation compared to non-gene-modified parental DCs.

Plasmid DNA vector

In the present experiment the construction of a nucleotide vector involves cloning of a gene of interest, i.e. e1a2 gene, into a bacterial plasmid vector, pVAX-1, under the control of a viral promoter. In addition, the plasmid DNA comprises a polyadenylation/transcription termination sequence. The plasmid is grown in bacteria (*E. coli*), purified, and dissolved in a saline solution. After injection, the plasmid DNA is taken up by host cells, where the encoded protein is made. In cell nuclei, the plasmid persists as a circular non-replicating episome and is not integrated into the host's genome.

Production of the minigene encoding the e1a2 fusion peptide started with a fill-in reaction (Pharmacia) using the following overlapping primers: 5'-TGCTAGCATGATCTGGCCCAACGACGGCGAGGGCGCCTTCCACGGCGACGC-CGAGGCCCTGCAGCGC-3' (see SEQ ID NO: 1) and 5'-AATCGATCACAGGCC-CTGGGGCTCGAAGTCGCTGGCCACGGGGCGCTGCAGGGC-3' (see SEQ ID NO: 2). The reaction mixture included, in addition to the two primers above, the Klenow fragment (*E. coli* DNA polymerase I), dNTPs and enzyme buffer,

according to the manufacture's recommendation. The reaction was carried out at room temperature for 1 h followed by PCR reaction using the same primers and by adding PCR buffer, Taq DNA polymerase, dNTPs and run for 95 °C 1 min, followed by 35 cycles of 95 °C 30 s, 65 °C 30 s and 72 °C 30 s. The obtained PCR fragment was initially cloned into pUC19 plasmid vector (New England BioLabs) at the Nhe I/Cla I site. The Nhe I/Cla I fragment was then recloned into pBK-CMV vector (Stratagene). A Nhe I/Kpn I fragment comprising the e1a2 minigene from the pBK-CMV was finally cloned into Nhe1 and Kpn1 site of the pVAX-1 vector (Invitrogen, CA, USA). Once incorporated into the vector, the e1a2 fusion gene is driven by the CMV promoter. The construct was confirmed by DNA sequencing. Large scale plasmid DNA purification was performed using the Qiagen Maxi-prep kit (Qiagen, Santas Clarita, CA, USA). Purity of plasmid DNA was determined by UV spectrophotometry and agarose gel electrophoresis. Purified DNA with an OD 260 nm/OD 280 nm absorbance ratio of greater than 1.9 was used. A portion of the pVAX-1 vector without and with the fusion gene sequence is illustrated in Fig. 8. The e1a2 fusion gene sequence and corresponding polypeptide sequence are found in SEQ ID NO: 3 and SEQ ID NO: 4, respectively.

The pVAX-e1a2 plasmid construct was tested for the production of correctly sized product by an *in vitro* transcription-coupled translation assay (TNT, Promega). Approximately 1 µg of plasmid DNA was incubated for 2 h at 30 °C in a 50 µl volume containing a mixture of 25 µl rabbit reticulocyte lysate, 2 µl of TNT reaction buffer, 1 µl of TNT T7 RNA polymerase, 1µl of a 1 mM amino acid mixture minus methion, 4 µl of (35S) methionine at 10 mCi/ml and 1 µl RNA RNAasin ribonuclease inhibitor at 40 U/µl. A 3µl volume of the reaction was loaded onto a 16 % SDS polyacrylamide gel. After drying, the gel was exposed to autoradiography film. The results are illustrated in Fig. 8, indicating presence of the e1a2 fusion protein in pVAX-e1a2 but not in wild type pVAX-1. In addition the transcript of pVAX-e1a2 was detected in transfected COS cells (data not shown). Thus, the results confirm that the

plasmid vector pVAX-e1a2 contains the 96 bp nucleotide sequence, which spans the fusion region of e1a2.

Vaccination of mice

For the tumorigenicity study, increasing doses of viable BM185wt cells were given subcutaneous (s.c.) to immunocompetent syngeneic mice. Injection of 500 cells into 6-8 weeks old mice caused 100 % mortality within a 3-4 week period.

A lethal dose of viable BM185wt parental tumor cells were injected subcutaneous (s.c.) into the right flank of each mouse on day 0. On day 7, 14 and 21 mice (5 mice/group) were immunized at the tumor site with either PBS saline solution, plasmid DNA, pVAX-1 or pVAX-e1a2, (100 µg/mouse) alone, DCs pulsed with the high score binding peptide, (SEQ ID NO: 5), (10^6 cells/10 µM peptide/mouse) or DCs mixed with pVAX-e1a2 DNA (10^6 cells/100µg plasmid DNA/mouse). In brief, 10^6 DCs were pulsed 2 h with 10 µM of peptide at 37 °C in FCS free medium in 5 % of CO_2. Cells were washed extensively before the immunization. One week after the last vaccination, tumor free mice were rechallenged with parental tumor cells (800 cells/mouse), s.c. at the left flank of each mouse. The tumor size was monitored twice per week. When the tumor is detectable, mice were considered to be at the end point of survival and were sacrificed.

As seen in Fig. 9, vaccination with neither pVAX-1, i.e. an empty plasmid DNA vector, pVAX-e1a2 alone, nor D2SC/CD40L cells protect animals. In contrast, vaccination with pVAX-e1a2 pre-mixed with D2SC/CD40L cells eliminates pre-existing tumor cells and protects mice from rechallenge with parental tumor cells.

Comparison of vaccination compositions

The vaccine composition of the present invention was compared with other vaccination strategies including DCs pulsed with tumor antigen peptide for treating mice with pre-existing bcr/abl positive tumors.

Mice (5 mice/group) were inoculated subcutaneous at right flank with 600 live BM185wt tumor cells on day 0. On day 7, 14 and 21, mice were immunized at the tumor site with (a) PBS; (b) pVAX-e1a2 (100 µg/mouse); (c) e1a2 peptide (AFHGDAEAL) (10 µM/mice); (d) D2SC/wt cells pulsed with e1a2 peptide; (e) D2SC/CD40L cells pulsed with e1a2 peptide; (f) D2SC/CD40L cells pulsed with control peptide 8 (HGDAEALQ); (g) D2SC/CD40L cells pulsed with tumor lysate derived from BM185 cells; (h) D2SC/CD40L cells mixed with pVAX-e1a2 plasmid DNA. One week after the last vaccination, tumor free mice were rechallenged with 800 live parental BM185wt tumor cells at left flank.

As seen in Fig. 10, the vaccine composition (D2SC/CD40L mixed with pVAX-e1a2, filled dots in Fig. 10) of the present invention is superior to other vaccination strategies, including D2SC/CD40L pulsed with the e1a2 peptide. Vaccination with either pVAX-e1a2, or e1a2 peptide or D2SC/CD40L cells pulsed with peptide 8, which has low affinity binding to MHC-I, failed to eradicate pre-existing leukemia and protect mice against challenge of parental tumor cells. Apparently, tumor antigens encoded by plasmid DNA and presented by DCs play an important role in the priming of a therapeutic immune response in this leukemia model. Functional expression of CD40L in DCs further enhances their ability to prime tumor specific T cell responses.

CTL assay

Splenocytes and lymph nodes (LN) from vaccinated mice were isolated. Percoll gradient-enriched T cells were re-stimulated for 5-7 days with BM185/wt (200 Gy, ^{137}Cs γ-radiation) or BM185/CD40L (200 Gy) in the presence of rhIL-2 (10 µg/ml). Viable cells were incubated for 4 h with pre-labeled target cells at various effector-to-target ratios. Target cells were pre-labeled with ^{51}Cr (25 µCi/10^6 cells) at 37 °C for 2 h. Cytolysis was measured by the release of ^{51}Cr into the medium and the results represent triplicate

samples. The percentage of specific lysis was calculated according to the following formula:

$$\text{percentage specific lysis} = 100 \times \frac{\text{experimental lysis - spontaneous lysis}}{\text{maximum lysis - spontaneous lysis}}$$

As seen in Figs. 11A and 11B, pre-mixing of plasmid DNA vector encoding the e1a2 fusion peptide and CD40L modified DCs induced a strong tumor-specific CTL response paralleled by a therapeutic response, and 80 % of these mice remained tumor-free for several months after tumor challenge ($p<0,01$). The *in vitro*-generated CTL response was directed against parental BM185 cells and the TAP deficient RMA-S cells pulsed with e1a2 peptide but not against A20 cells.

Activation of e1a2-specific T cells

CD8+ T cells were isolated from mice immunized with (a) PBS; (b) e1a2 peptide (10 µM/mice); (c) pVAX-e1a2 (100 µg/mouse); (d) D2SC/CD40L cells pulsed with e1a2 peptide; (e) D2SC/CD40L cells pulsed with tumor lysate derived from BM185 cells; (f) D2SC/CD40L cells mixed with pVAX-e1a2 plasmid DNA and restimulated with irradiated BM185 cells after pulsing with e1a2 peptide. T cells with e1a2 specificity are shown to bind to the H-2Ld:Ig/e1a2 complex. These e1a2 specific T cells recognize tumor cells and thus kill them *in situ*. The percentage of e1a2 specific T cells from the differently immunized mice is summarized in Fig. 12.

Together, these results clearly prove that vaccine composition of present invention eliminates the pre-existing tumor cells and protects mice against rechallenge of tumor cells.

Summarizing the experiments, a novel vaccination composition and strategy to enhance the DNA vaccination against pre-existing bcr/abl tumor using genetically modified DCs is presented. A pre-mixed combination of genetically modified DCs and DNA vector induces *bcr/abl* specific immune

response. The vaccine composition was effective in eliminating pre-existing *bcr/abl* positive tumor and protecting animal from rechallenge of viable parental tumor. The composition induced a strong tumor-specific and e1a2-specific CTL response paralleled by a therapeutic response, and 60-80 % of these mice remained tumor-free for several months after tumor challenge at a distant site. Thus, the present vaccine composition is well adapted as therapeutic composition for Philadelphia chromosome-positive leukemia.

It will be understood a person skilled in the art that various modifications and changes may be made to the present invention without departure from the scope thereof, which is defined by the appended claims.

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CLAIMS

1. A nucleotide vaccine composition comprising:
   - nucleotide sequence encoding an antigen; and
   - antigen presenting cells modified for expression of an immune response effecting molecule.

2. The vaccine composition according to claim 1, wherein said vaccine is provided as a mixture of said nucleotide sequence and said antigen presenting cells.

3. The vaccine composition according to claim 1, wherein said antigen presenting cells are selected from at least one of:
   - dendritic cells;
   - macrophages;
   - monocytes; and
   - B cells.

4. The vaccine composition according to claim 1, wherein said immune response effecting molecule is encoded by a gene sequence cloned into said antigen presenting cells, said gene sequence is selected from at least one of:
   - cytokine gene;
   - adhesion molecule gene;
   - interferon gene; and
   - chemokine and chemokine receptor gene.

5. The vaccine composition according to claim 1, wherein said nucleotide sequence is provided in a vector selected from at least one of:
   - plasmid; and
   - viral vector.

6. The vaccine composition according to claim 5, wherein said vector comprises an immune response effecting nucleotide sequence.

7. The vaccine composition according to claim 6, wherein said immune response effecting nucleotide sequence is an unmethylated cytidine phosphate guanosine sequence.

8. The vaccine composition according to claim 1, further comprising a carrier solution in turn comprising a second immune response effecting molecule selected from at least one of:

- cytokine;
- adesion molecule;
- interferon; and
- chemokine.

9. A method of producing a nucleotide vaccine composition based on mixing a nucleotide sequence encoding an antigen and antigen presenting cells modified for expression of an immune response effecting molecule.

10. The method according to claim 9, further comprising the steps of:

- cloning said nucleotide sequence encoding said antigen into a vector; and
- propagating said vector in a propagation system.

11. The method according to claim 9, further comprising the steps of:

- isolating said antigen presenting cells from a subject; and
- cloning a gene sequence into said antigen presenting cells, said gene sequence encoding said immune response effecting molecule.

12. The method according to claim 11, wherein said gene sequence is selected from at least one of:

- cytokine gene;
- adhesion molecule gene;
- interferon gene; and
- chemokine and chemokine receptor gene.

13. The method according to claim 9, further comprising the step of adding additional substance having immune response effecting properties, said additional substance is selected from at least one of:

- cytokine;
- adesion molecule;
- interferon; and
- chemokine.

14. A method of producing an immune response comprising the step of administering to a subject a vaccine composition according to claim 1.

15. A method of treating or preventing a disease in a subject comprising the step of administering to said subject a vaccine composition according to claim 1, said antigen being associated with an agent causing said disease.

16. The method according to claim 14 or 15, wherein said antigen presenting cells are adapted for presenting at least a fragment of said antigen to cells of the immune system of said subject.

17. The method according to claim 14 or 15, wherein said subject is a mammalian subject.

18. The method according to claim 17, wherein said mammalian subject is a human subject.

19. The method according to claim 15, wherein said disease is selected from at least one of:

- infectious disease;
- cancer;
- allergy; and
- diabetes.

ABSTRACT

The present invention refers to a novel nucleotide vaccine composition and use thereof for treating and/or preventing different diseases, including infectious diseases, cancer, allergy and diabetes. The vaccine composition comprises a nucleotide sequence encoding an antigenic molecule displaying antigenicity of an agent that causes the disease and gene-modified antigen presenting cells (APCs), preferably provided as an intermixture. The APCs are modified for expressing immunopotentiating molecules, co-stimulating the immune response of the subject receiving the vaccine composition.

(Fig. 1)

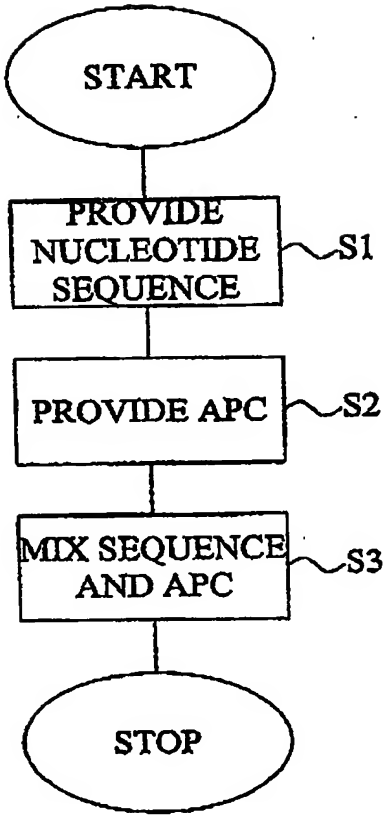

START
PROVIDE NUCLEOTIDE SEQUENCE
S1
PROVIDE APC
S2
MIX SEQUENCE AND APC
S3
STOP

FIGURE 1

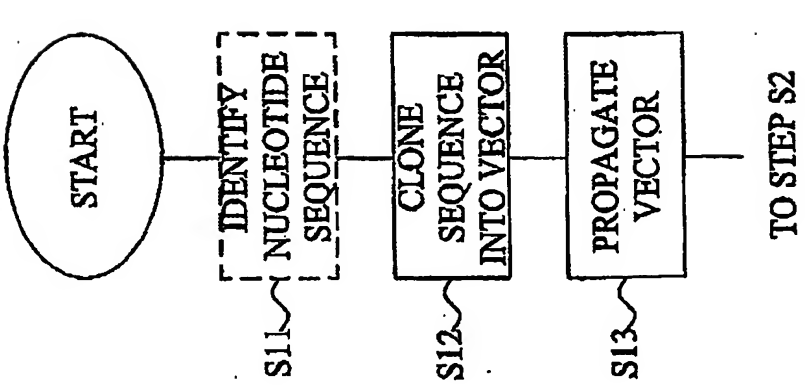


FIGURE 2

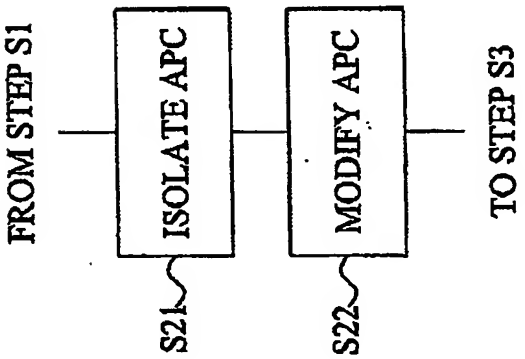


FIGURE 3

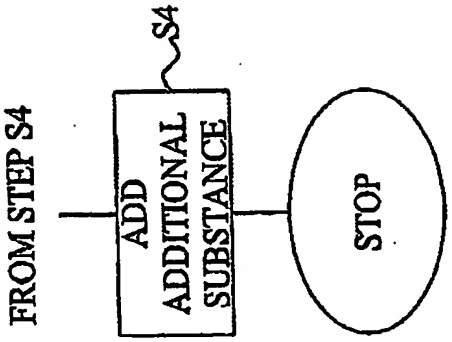


FIGURE 4

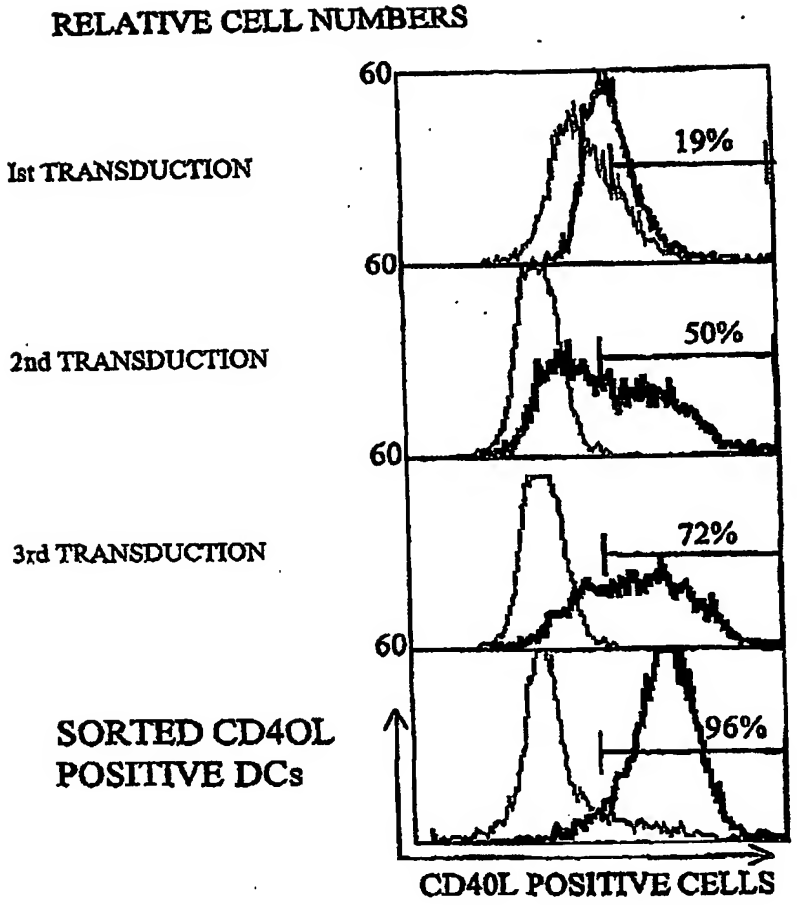

RELATIVE CELL NUMBERS
1st TRANSDUCTION
2nd TRANSDUCTION
3rd TRANSDUCTION
SORTED CD40L POSITIVE DCs
60
60
60
60
19%
50%
72%
96%
CD40L POSITIVE CELLS

FIGURE 5

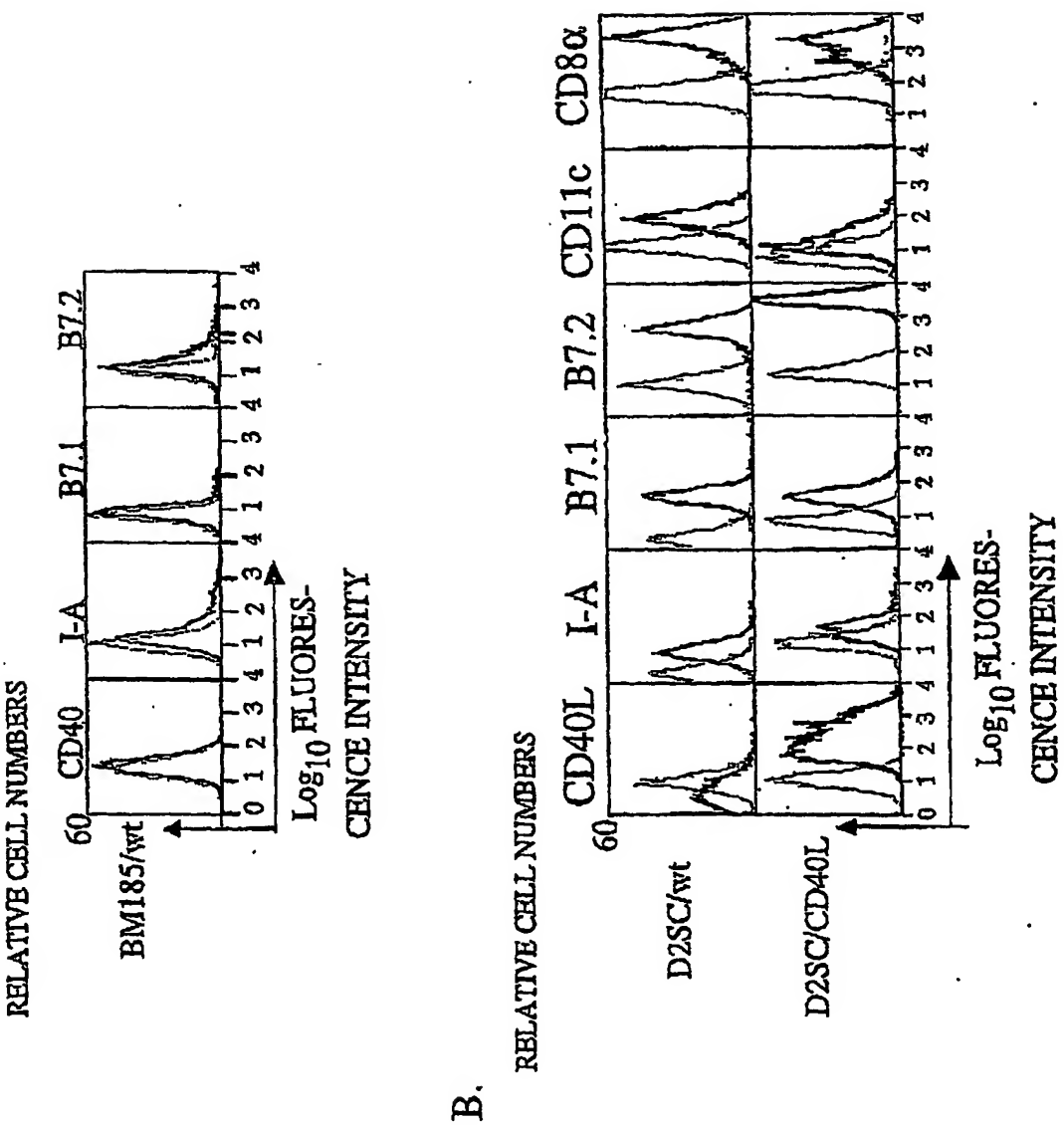

A.
RELATIVE CELL NUMBERS
BM185/wt
CD40
I-A
B7.1
B7.2
60
0 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
Log10 FLUORES-
CENCE INTENSITY
B.
RELATIVE CELL NUMBERS
D2SC/wt
D2SC/CD40L
CD40L
I-A
B7.1
B7.2
CD11c
CD8α
60
0 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
Log10 FLUORES-
CENCE INTENSITY

FIGURE 6

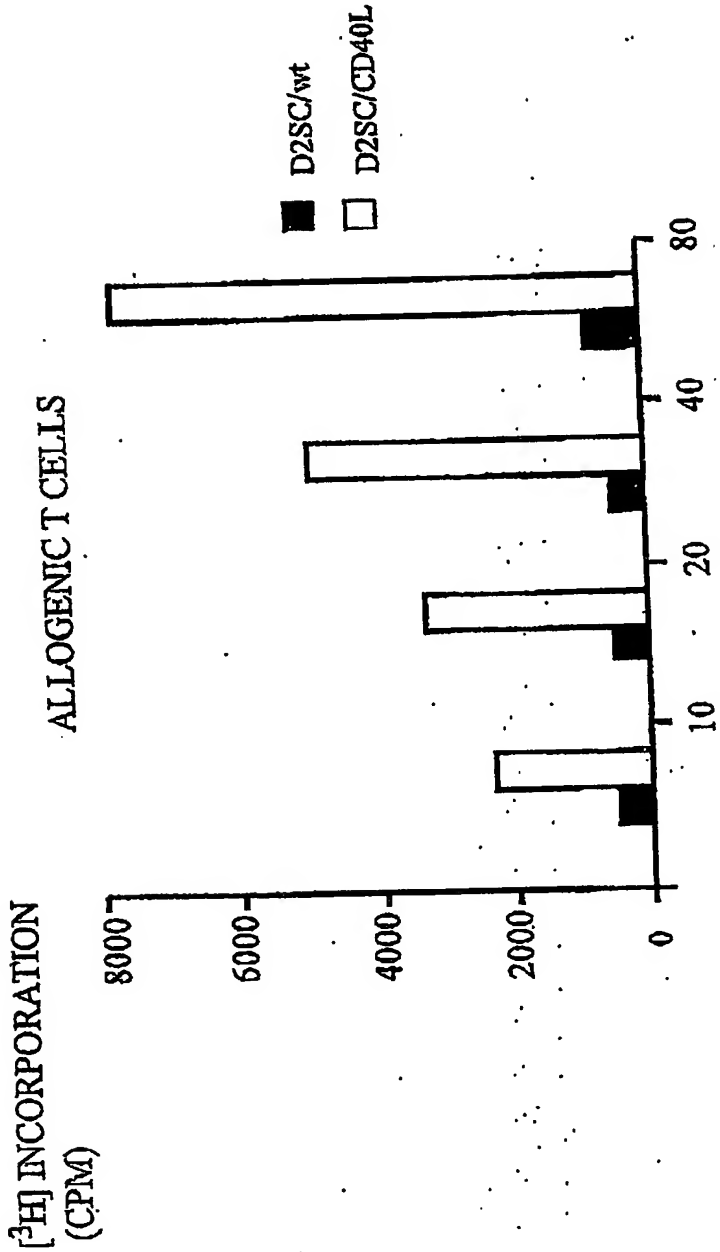

D2SC/wt
D2SC/CD40L
ALLOGENIC T CELLS
[3H] INCORPORATION (CPM)
8000
6000
4000
2000
0
10
20
40
80
S:R RATION

FIGURE 7A

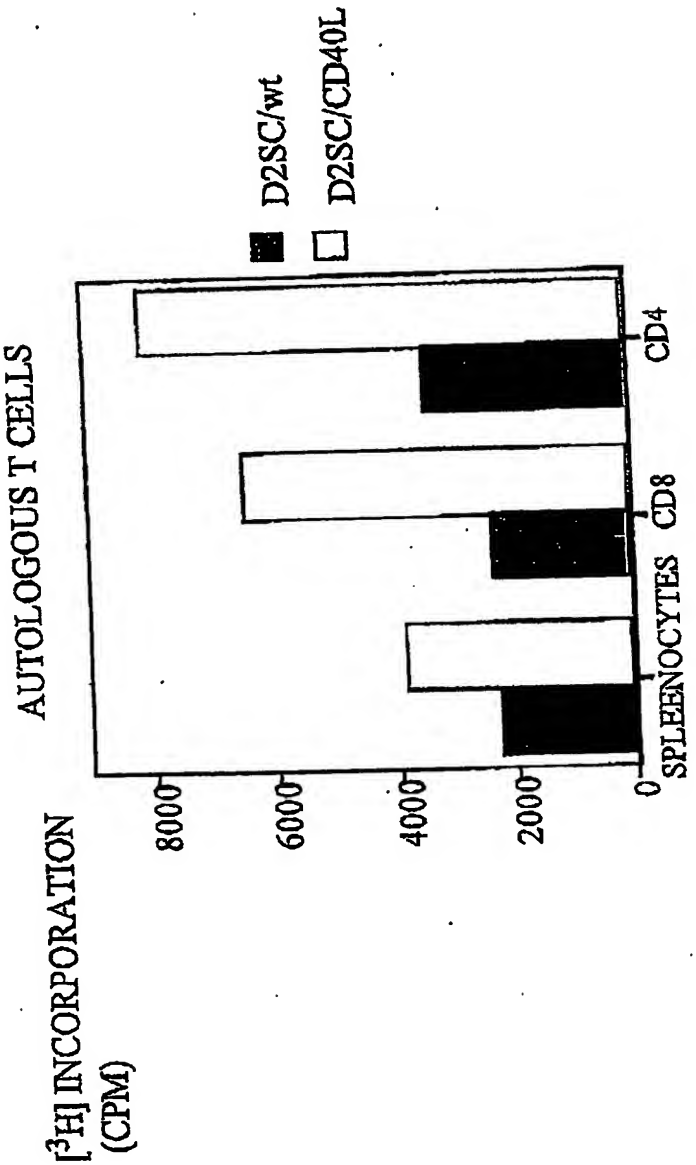
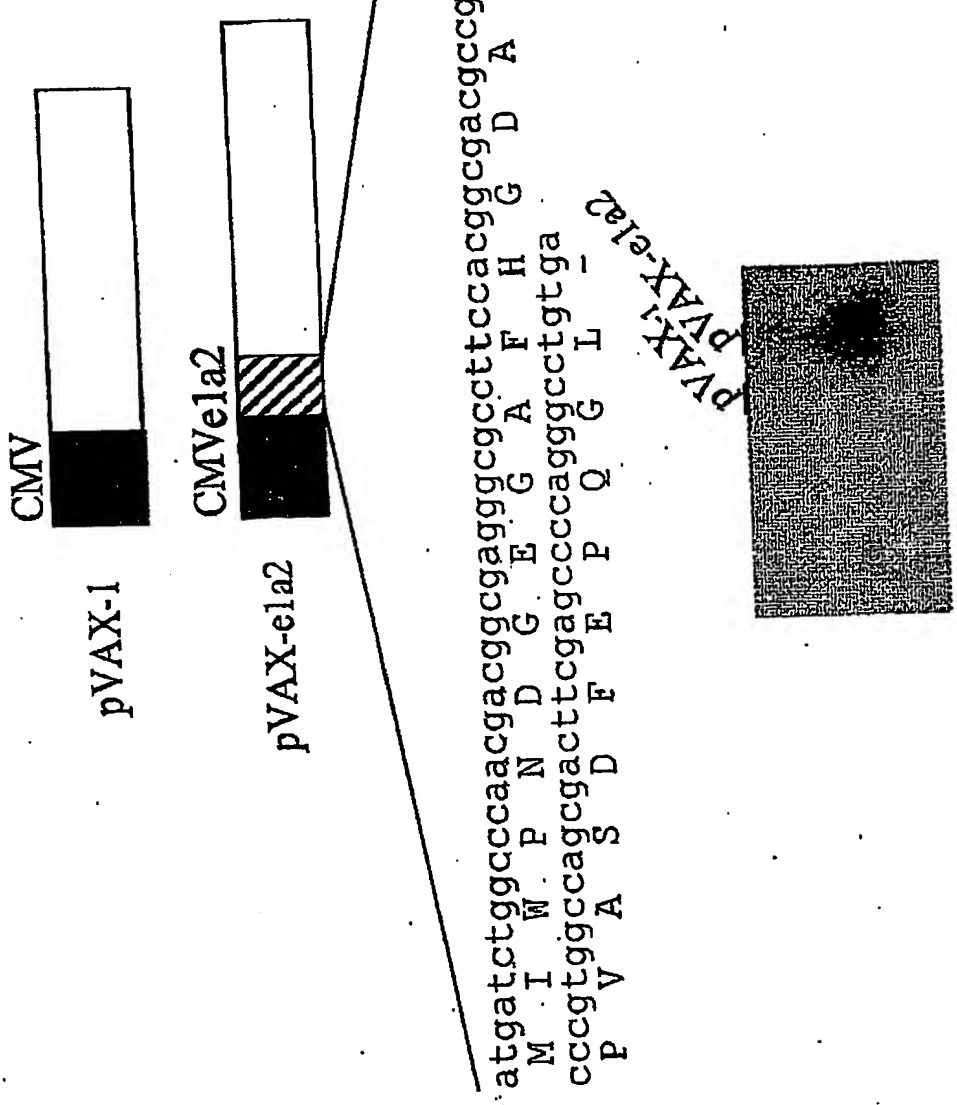

D2SC/wt
D2SC/CD40L
AUTOLOGOUS T CELLS
[3H] INCORPORATION (CPM)
8000
6000
4000
2000
0
CD4
CD8
SPLEENOCYTES
S:R RATIO=1:10

FIGURE 7B


CMV
CMVe1a2
pVAX-1
pVAX-e1a2
pVAX-e1a2
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M I W P N D G E G A F H G D A E A L Q R
ccgtggccagcgacttcgagccccagggcctgtga
P V A S D F E P Q G L –

FIGURE 8

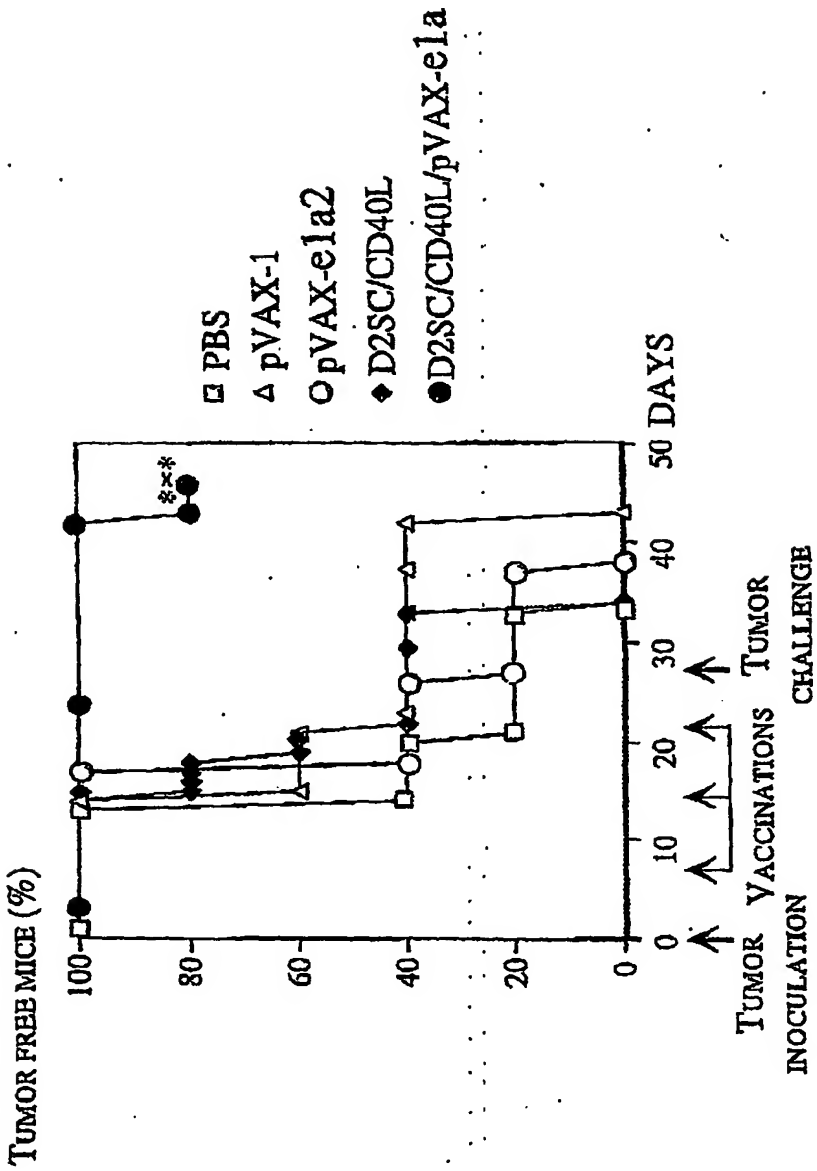
PBS
pVAX-1
pVAX-e1a2
D2SC/CD40L
D2SC/CD40L/pVAX-e1a
***
TUMOR FREE MICE (%)
100
80
60
40
20
0
0
10
20
30
40
50 DAYS
TUMOR INOCULATION
VACCINATIONS
TUMOR CHALLENGE

FIGURE 9

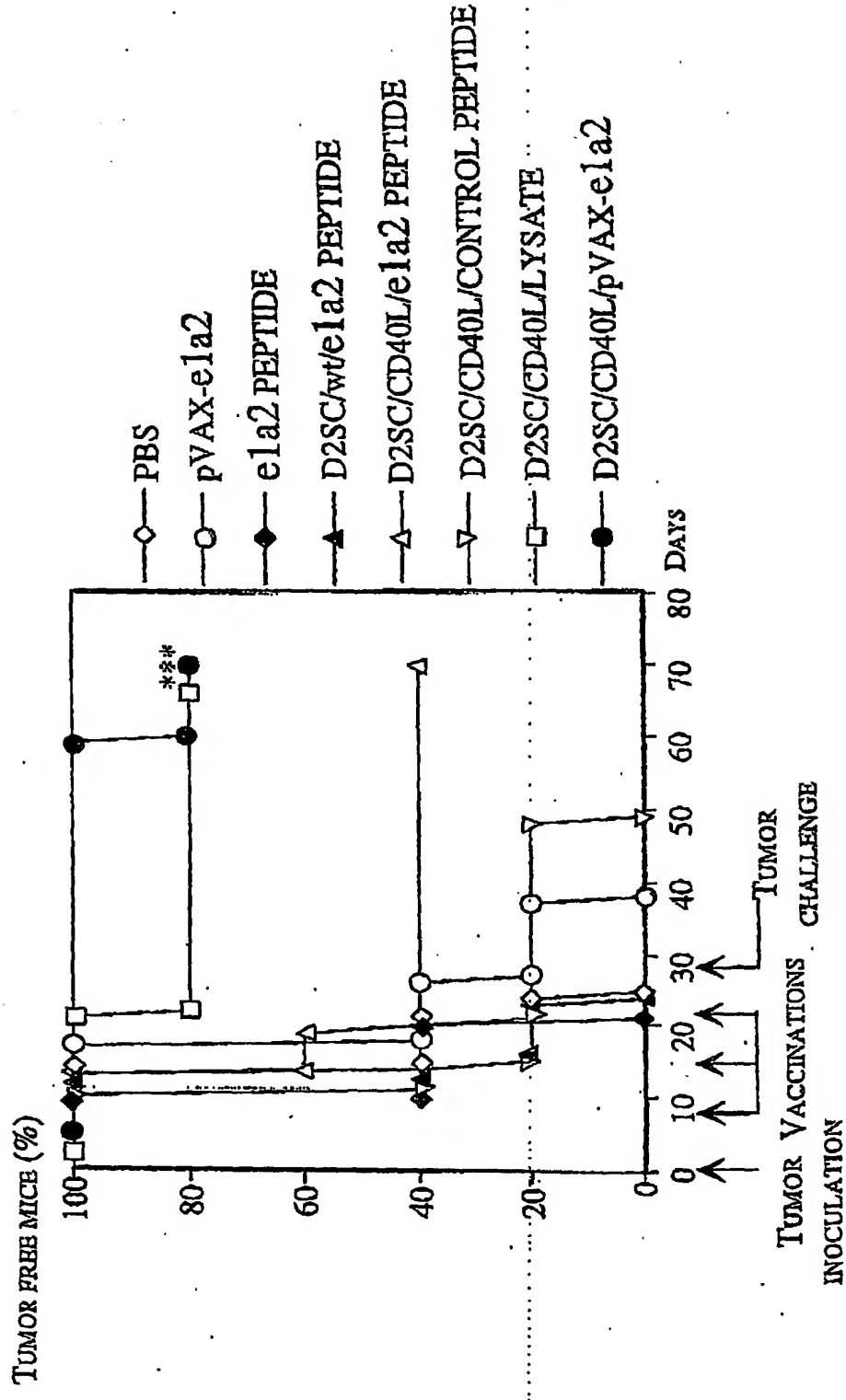
PBS
pVAX-e1a2
e1a2 PEPTIDE
D2SC/wt/e1a2 PEPTIDE
D2SC/CD40L/e1a2 PEPTIDE
D2SC/CD40L/CONTROL PEPTIDE
D2SC/CD40L/LYSATE
D2SC/CD40L/pVAX-e1a2
***
TUMOR FREE MICE (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
DAYS
TUMOR VACCINATIONS
TUMOR CHALLENGE
INOCULATION

FIGURE 10

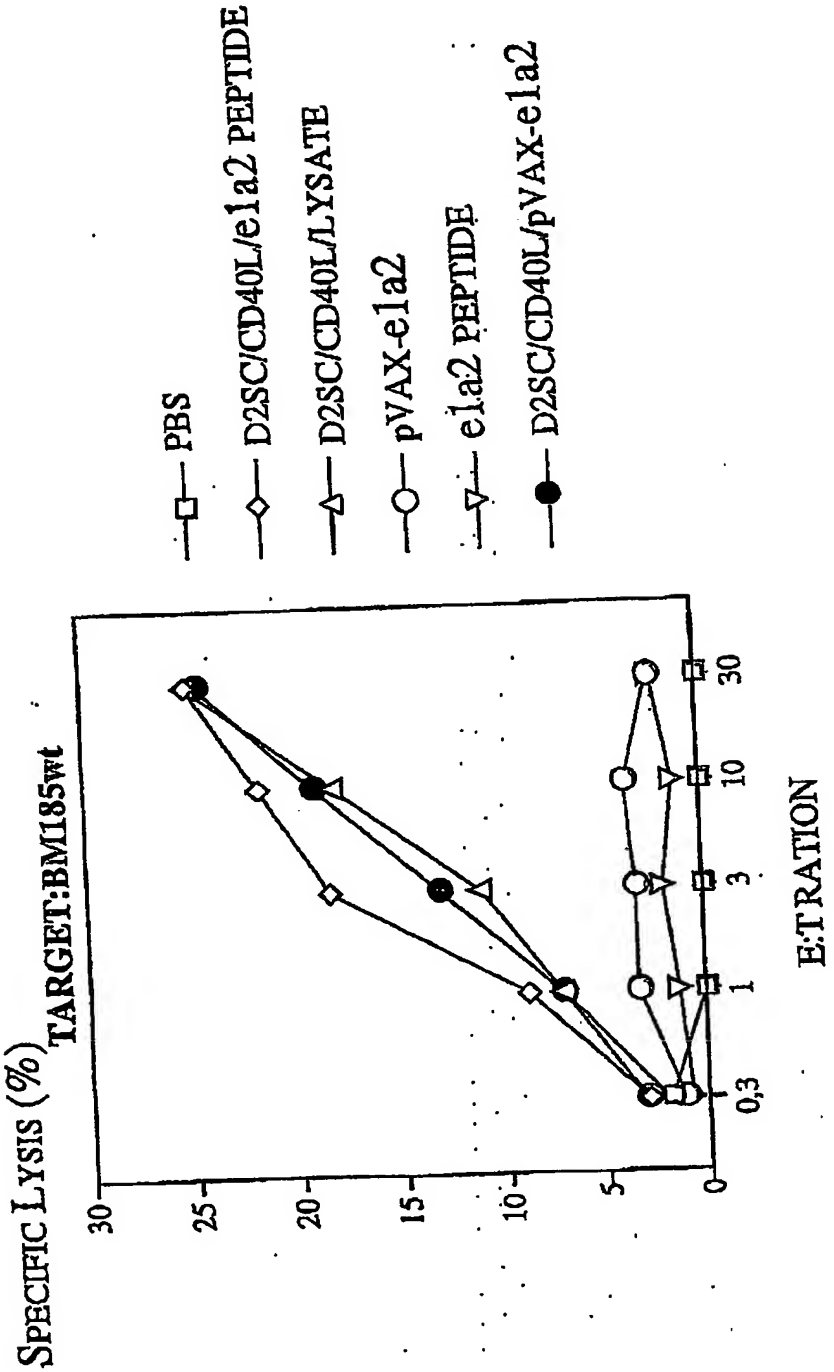
PBS
D2SC/CD40L/e1a2 PEPTIDE
D2SC/CD40L/LYSATE
pVAX-e1a2
e1a2 PEPTIDE
D2SC/CD40L/pVAX-e1a2
SPECIFIC LYSIS (%)
TARGET:BM185wt
30
25
20
15
10
5
0
0,3
1
3
10
30
E:T RATION

FIGURE 11A

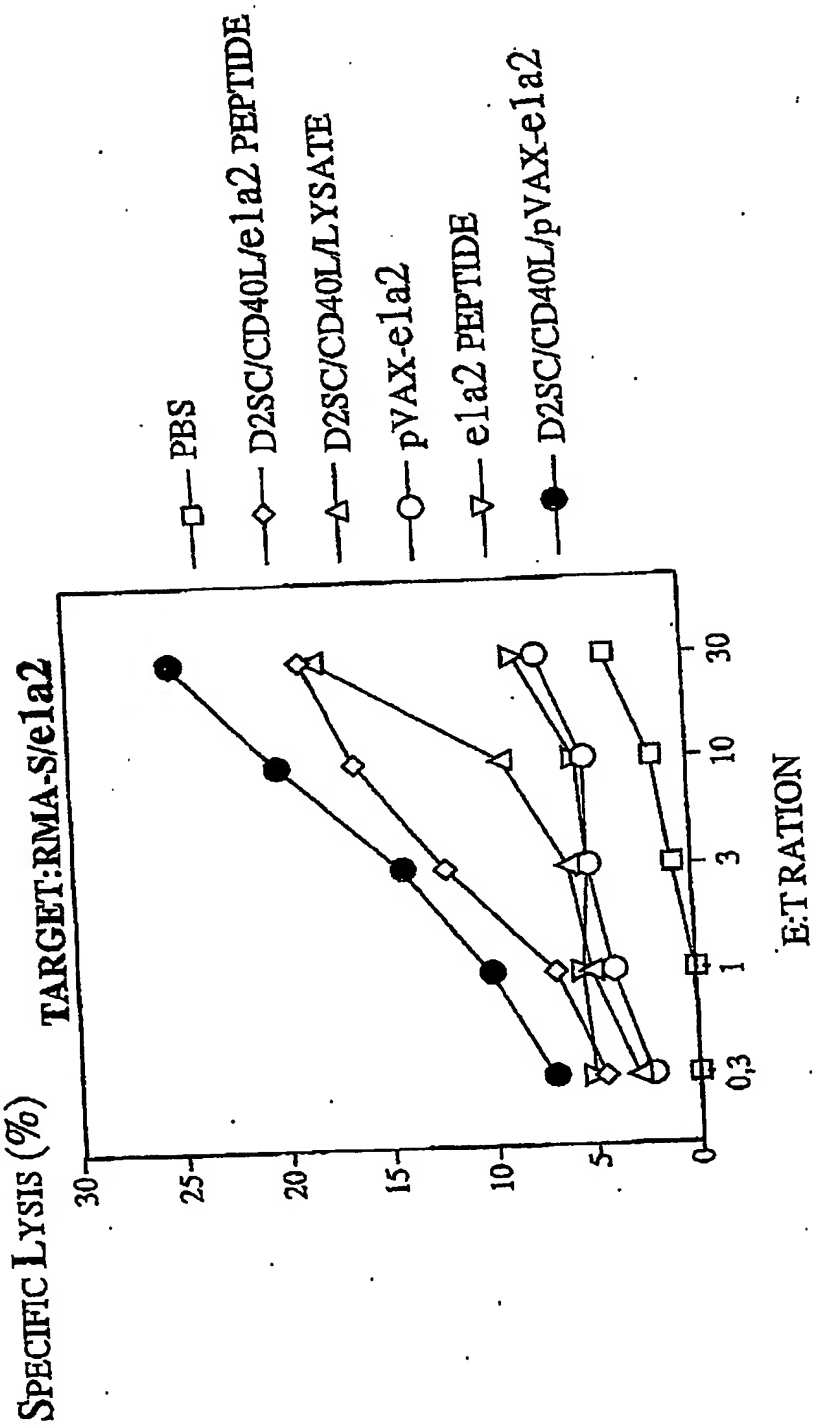
PBS
D2SC/CD40L/e1a2 PEPTIDE
D2SC/CD40L/LYSATE
pVAX-e1a2
e1a2 PEPTIDE
D2SC/CD40L/pVAX-e1a2
SPECIFIC LYSIS (%)
TARGET:RMA-S/e1a2
30
25
20
15
10
5
0
0,3
1
3
10
30
E:T RATION

FIGURE 11B

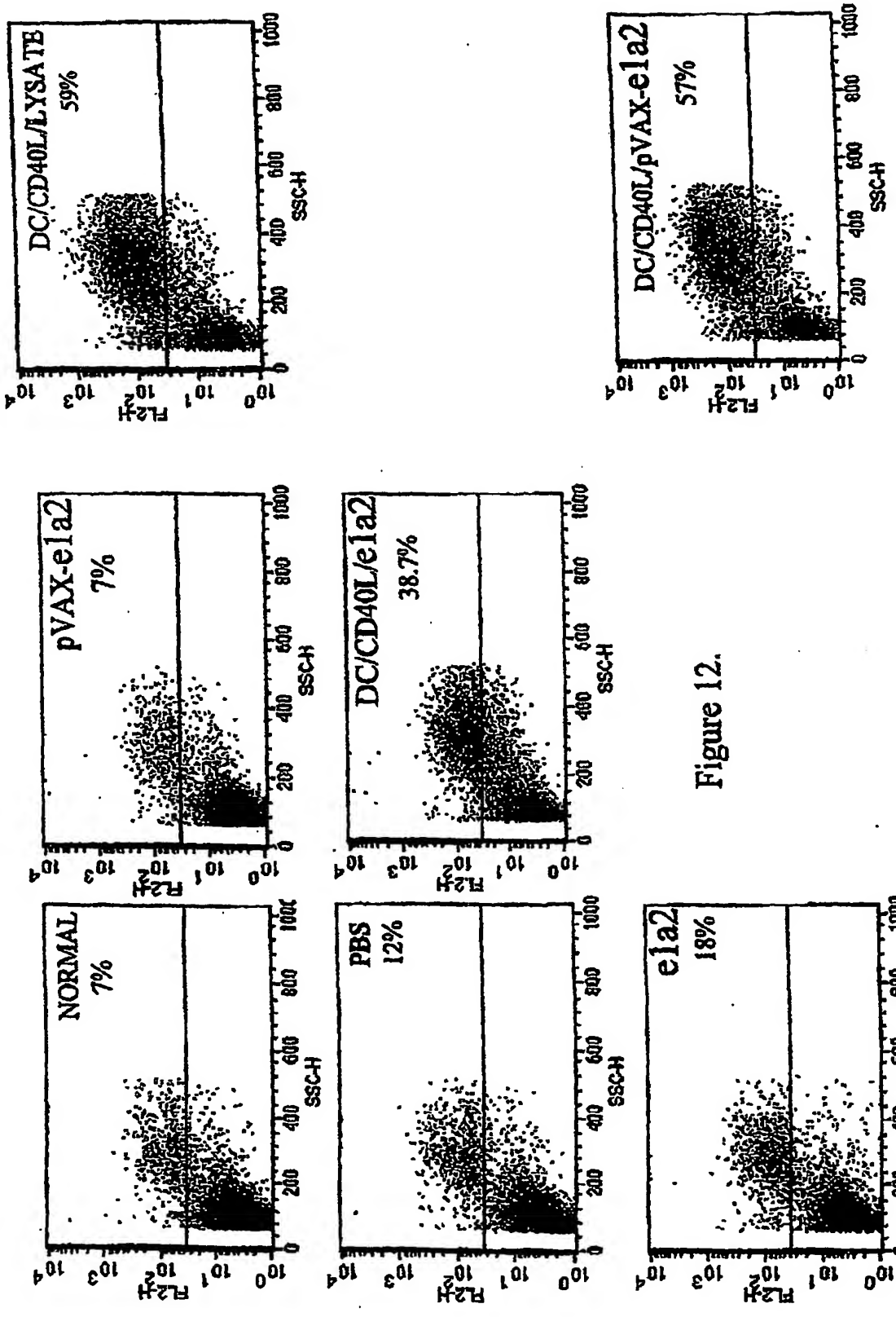
DC/CD40L/LYSATE
59%
DC/CD40L/pVAX-e1a2
57%
pVAX-e1a2
7%
DC/CD40L/e1a2
38.7%
NORMAL
7%
PBS
12%
e1a2
18%
SSC-H
FL2-H

Figure 12.

sekvenslista.ST25
SEQUENCE LISTING

<110> Huang, Mallen

<120> Nucleotide vaccine composition

<130> P366SE00

<160> 7

<170> PatentIn version 3.1

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gcagcgc 67

<210> 2
<211> 54
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<400> 2
aatcgatcac aggccctggg gctcgaagtc gctggccacg gggcgctgca gggc 54

<210> 3
<211> 96
<212> DNA

sekvenslista.ST25

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<220>

<221> CDS

<222> (1)..(96)

<223>

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Met Ile Trp Pro Asn Asp Gly Glu Gly Ala Phe His Gly Asp Ala Glu
1 5 10 15

gcc ctg cag cgc ccc gtg gcc agc gac ttc gag ccc cag ggc ctg tga 96
Ala Leu Gln Arg Pro Val Ala Ser Asp Phe Glu Pro Gln Gly Leu
20 25 30

<210> 4

<211> 31

<212> PRT

<213> Homo sapiens

<400> 4

Met Ile Trp Pro Asn Asp Gly Glu Gly Ala Phe His Gly Asp Ala Glu
1 5 10 15

Ala Leu Gln Arg Pro Val Ala Ser Asp Phe Glu Pro Gln Gly Leu
20 25 30

<210> 5

<211> 9

<212> PRT

<213> Homo sapiens

<400> 5

Ala Phe His Gly Asp Ala Glu Ala Leu
1 5

<210> 6

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic peptide with low binding to mouse MHC-I antigen (H-2Kd)

<400> 6

His Gly Asp Ala Glu Ala Leu Gln
1 5

<210> 7

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic peptide that does not bind to mouse MHC-I antigen (H-2K d)

<400> 7

Ala Thr Gly Phe Lys Gln Ser Ser Lys
1 5

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